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IN VITRO CULTIVATION OF SCHIZONTS OF *SARCOCYSTIS SPEERI* DUBEY AND LINDSAY, 1999

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ABSTRACT: Schizonts of *Sarcocystis speeri* Dubey and Lindsay, 1999 were cultured in vitro in bovine monocyte and equine kidney cell cultures inoculated with infected tissues of nude and γ-interferon knockout mice fed sporocysts from opossums, *Didelphis albiventris.* At least 1 asexual cycle was completed in 3 days. In vitro-grown merozoites were structurally and antigenically distinct from those of *Sarcocystis neurona* and *Sarcocystis falcatula.* Culture-derived merozoites of *S. speeri* were not infective to budgerigars (*Melopsittacus undulatus*).

The North American opossum (*Didelphis virginiana*) is a host for at least 3 pathogenic species of *Sarcocystis, Sarcocystis falcatula* of avians (Box and Duszynski, 1978; Box et al., 1984), *Sarcocystis neurona* of horses (Dubey et al., 1991; Fenger et al., 1997; Dubey and Lindsay, 1998), and *Sarcocystis speeri* with an unknown intermediate host (Dubey et al., 1998; Dubey and Lindsay, 1999). Of these, only *S. falcatula* and *S. neurona* have been cultured in vitro (Dubey et al., 1991; Marsh et al., 1997; Lindsay et al., 1999). We report in vitro cultivation of *S. speeri.*

MATERIALS AND METHODS

Donor mice

Tissues from 3 (2 nude, 1 γ-interferon knockout [KO]) donor mice were used for the present investigation. Two nude mice (nos. 4308, 4309) had been fed sporocysts from opossum no. 2 (*Didelphis albiventris*) from Argentina as described (Dubey, Venturini, Venturini and Speer, 2000). Mouse no. 4308 was killed when it showed signs of illness at 33 days postfeeding (DPF) and mouse no. 4309 died 39 DPF. The liver from mouse no. 4309 was kept overnight at 4 C before examination. The γ-interferon KO mouse no. 4551 was killed 29 DPF sporocysts from an experimentally infected opossum no. 25 (Dubey, Speer et al., 2000). Opossum no. 25 had been fed sarcocysts of *S. speeri* from KO mice that had been fed sporocysts from a naturally infected Argentinian opossum no. 1 (Dubey, Venturini, Venturini, and Speer, 2000). Thus, all 3 donor mice had been infected with *S. speeri* originally derived from *D. albiventris* from Argentina.

Livers from nude mice nos. 4308 and 4309 and the brain of KO mouse no. 4551 were homogenized in Hank's balanced salt solution (HBSS) for inoculation into mice or cell cultures. Smears made from the tissues of the same 3 mice were stained with Giemsa and examined microscopically.

In vitro cultivation in flasks

For in vitro cultivation, liver homogenate from mouse no. 4309 was inoculated onto bovine monocyte (M617) cultures (Speer et al., 1985) and subcultured onto equine kidney (EK) cells (Dubey, Mattson et al., 1999) (culture A). Brain homogenate from KO mouse no. 4551 was inoculated onto EK cells (culture B). Cultures were maintained in RPMI culture medium as described (Dubey, Mattson et al., 1999) and subcultured in EK or M617 cells.

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Development in fixed coverslip cultures

The first and second passages of *S. speeri* merozoites from culture A were grown further in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL 70, American Type Culture Collection, Rockville, Maryland). The CV-1 cells were grown to confluence in 25-cm^2 plastic cell culture flasks in growth media consisting of 10% (v/v) fetal bovine serum (FBS) in RPMI-1640 medium supplemented with 100 U penicillin G/ml and 100 mg streptomycin/ml. Cell cultures were maintained in growth medium in which the FBS content was lowered to 2%. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2 and 95% air. For descriptive studies, merozoites from a 29-day culture in M617 cells were harvested from infected cell cultures by removing the medium and replacing it with HBSS without calcium and magnesium. This cell mixture was passed through a 27-gauge needle attached to a 10-ml syringe to rupture host cells, filtered through a sterile 3-μm filter to remove cellular debris, and the number of merozoites in the filtrate was determined using a hemacytometer. Monolayers of CV-1 cells on 22-mm^2 glass coverslips in 6-welled tissue culture plates were inoculated with 2×10^5 or 5×10^5 merozoites.

Coverslips with infected monolayers were removed on 1, 2, 3, and 4 days postinoculation (PI), fixed in 10% phosphate-buffered formalin for 30 min, placed in 100% methanol for 10 min, stained with a Giemsa type stain, attached to glass microscope slides with Permount (Fisher Scientific, Fair Lawn, New Jersey), and examined by light microscopy. Parasites were measured with a calibrated ocular micrometer.

Growth in live double coverslip preparations

Infected EK cells from culture B were trypsinized, centrifuged, and resuspended in culture medium containing 10% FBS, inoculated into 24-well culture plates (Costar) containing coverslips, and incubated at 38 C, 5% CO_2, 95% air (Dubey, Mattson et al., 1999). Cultures were examined by phase-contrast microscopy in double coverslip preparations (Parker, 1962) at 1–10 days after passage (110–119 days after the original isolation of the parasite in cell culture).

Infectivity of *S. speeri*-infected tissues to KO mice

Homogenates of livers from nude mice nos. 4308 and 4309 were inoculated subcutaneously (s.c.) into 5 KO mice (3 with liver of 4308 and 2 with liver of 4309).

Infectivity of cultured merozoites to mice

Merozoites (10^5–5×10^5) from cultures A and B were inoculated into 6 KO mice, 4 mice with culture A and 2 mice with culture B. The KO mice (BALB/c-IfnG^{tmits}) were obtained from Jackson Laboratories, Bar Harbor, Maine.

Infectivity of *S. speeri* merozoites to budgerigars (*Melopsittacus undulatus*)

Merozoites (1.4×10^6/animal) from a 93-day culture of *S. speeri* in EK cells (culture B) were inoculated s.c. into 4 budgerigars (nos. 93–96). The birds were obtained from a local aviary (Dubey and Lindsay, 1998). The birds were killed 28 DPF.

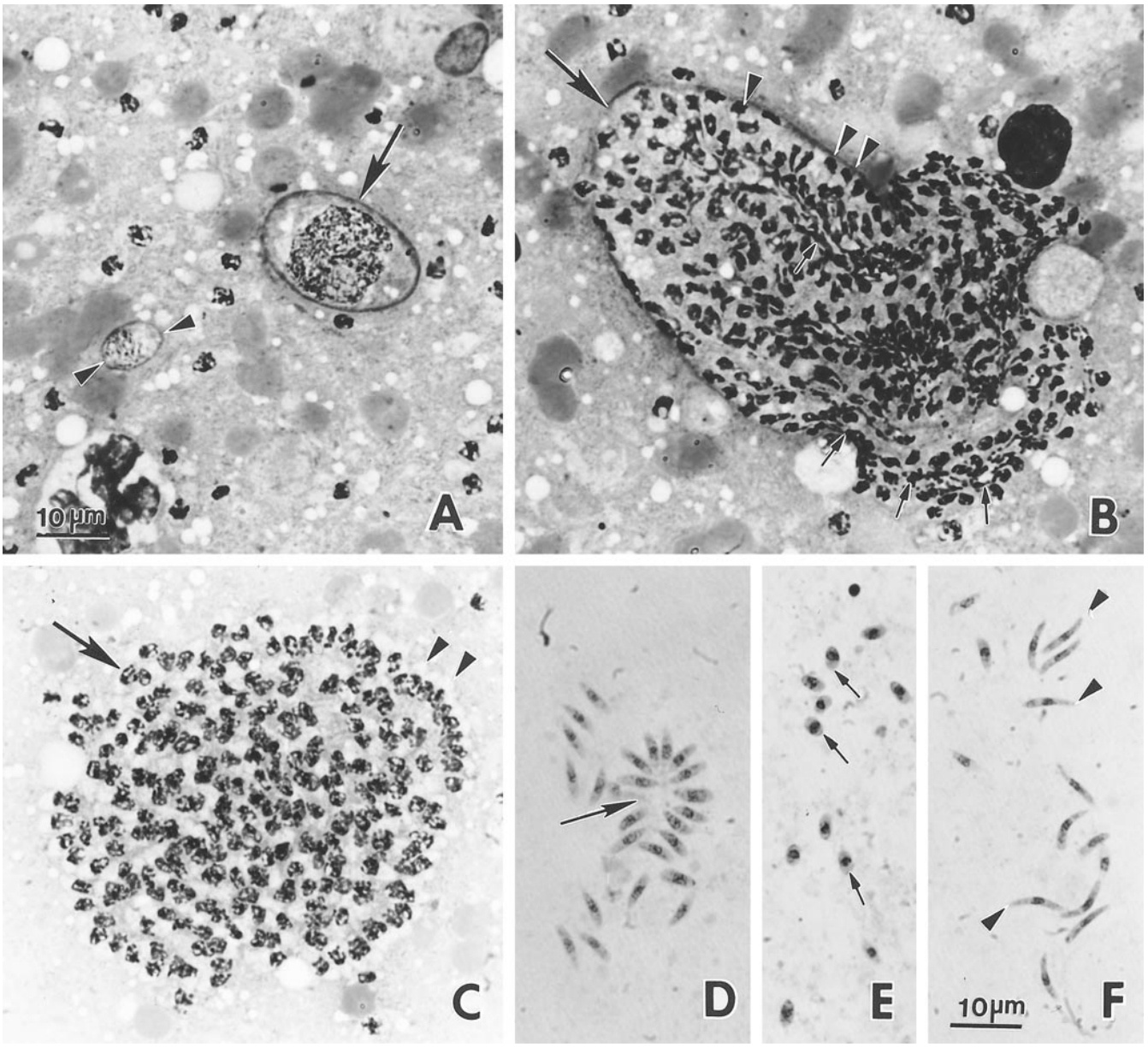

FIGURE 1. *Sarcocystis speeri* stages in imprints of the liver of KO mouse 4309. Giemsa stain. Bar = 10 μm and applies to all figures. A–C, methanol fixed; D–F, formalin fixed and postfixed in methanol. **A.** Young schizont (opposing arrowheads) and an intermediate-sized schizont (arrow). Numerous merozoites are scattered in the background. **B.** Large schizont (arrow) with >100 nuclear lobes attached by threadlike material (small arrows). Merozoites (arrowheads) are forming at the periphery. **C.** Large schizont (arrow) with many nuclei. Note few merozoites forming at the periphery. **D–F.** Different sized merozoites all in the same smear. Note the slender (arrowheads) and stubby merozoites (small arrows) and merozoites in a rosette (large arrow).

Histologic and immunohistochemical staining

Tissues from most internal organs of mice and birds were fixed in 10% buffered neutral formalin. Paraffin sections were cut at 5 μm thickness and examined microscopically after staining with hematoxylin and eosin stain. Deparaffinized sections were reacted with anti-*S. neurona*, anti-*S. falcatula* (Lindsay and Dubey, 1989; Dubey and Lindsay, 1998; Dubey, Mattson et al., 1999), and anti-*S. speeri* antibodies (Dubey and Lindsay, 1999). The peroxidase–anti-peroxidase system was used for avian tissues and the avidin–biotin system was used for murine tissues. Tissues from budgerigars infected with *S. falcatula* and mice infected with *S. neurona* and *S. speeri* were used as controls (Dubey and Lindsay, 1998, 1999).

RESULTS

Development of *S. speeri* in the liver of mice

Merozoites were seen in impression smears from the brain of KO mouse no. 4551 and livers of nude mice nos. 4308 and 4309. Developing schizonts with fully formed merozoites were seen (Fig. 1). At least 2 sizes of merozoites were seen in Giemsa-stained smears (Fig. 1). The slender merozoites were 8–10 μm long, and short merozoites were 4–5 μm long (Fig. 1D–F).

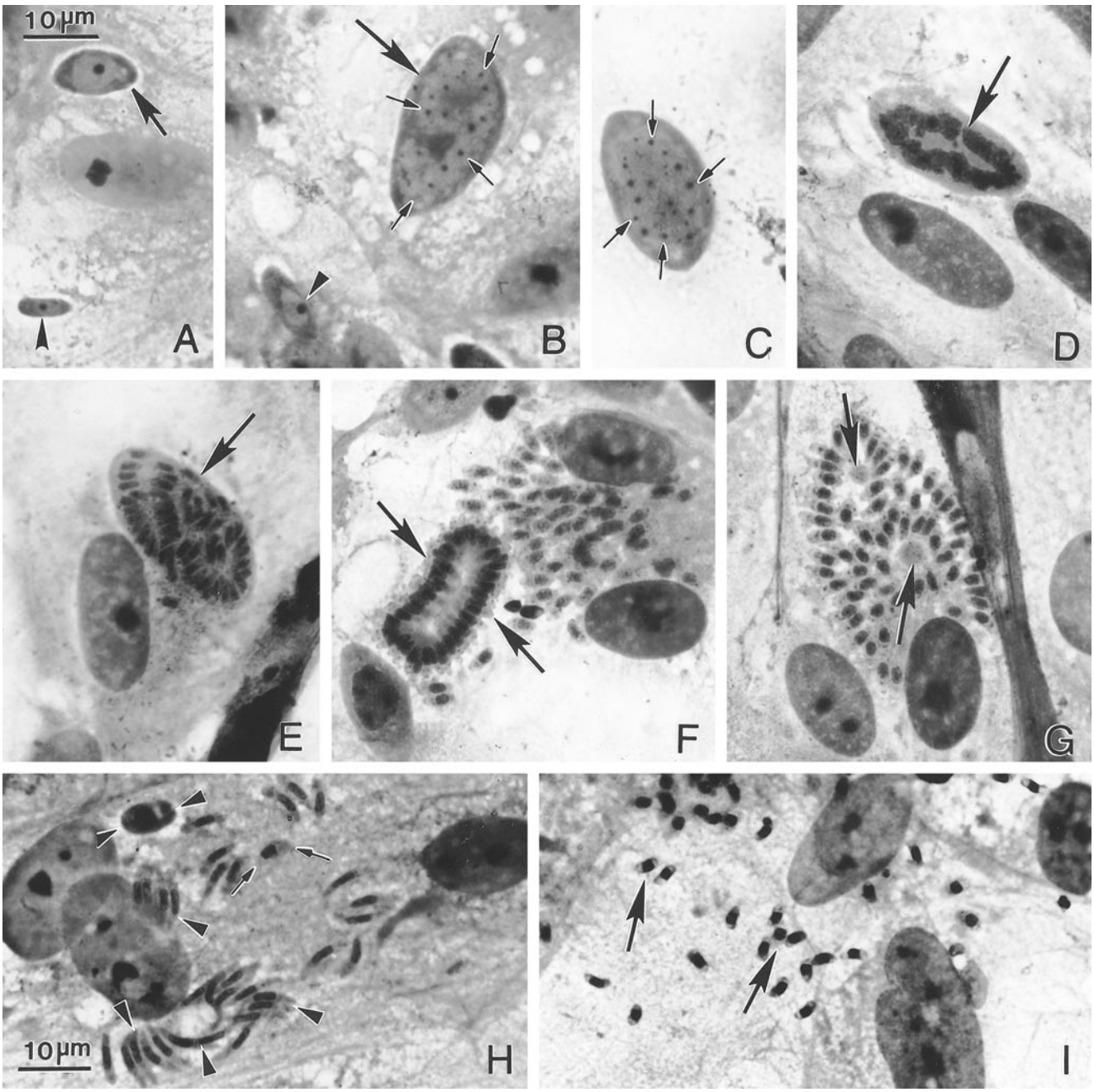

FIGURE 2. Asexual development of *S. speeri* in monkey kidney cells. Formalin and methanol fixed, Giemsa stain. All figures are at the same magnification. **A.** Young elongated meront (arrowhead) with central nucleus and a developing schizont with irregularly shaped nucleus (arrow). **B.** Schizont with morula-shaped nucleus (large arrow) containing several nucleoli (small arrows). An elongated schizont with a nucleolus in the nucleus (arrowheads) is also present. **C.** Schizont with nucleus containing several nucleoli (arrows). **D.** Schizont (arrow) with many nuclear lobes. The nucleoli have disappeared. **E.** Schizont with early stage of merozoite formation. Merozoites are forming in rows throughout the schizont. **F.** Schizont with all peripherally arranged merozoites (arrows). **G.** Schizonts with residual bodies (arrows). Different sized merozoites from the same coverslip preparation. Note elongated slender (arrowheads) and stubby merozoites (arrows) and possibly 2 developing schizonts (opposing arrowheads and arrows).

In vitro cultivation in flasks

Motile merozoites were seen on the day of inoculation and 1 day PI in M617 cells inoculated with liver homogenate of mouse no. 4309. Organisms were subcultured (passage 1) successfully in EK cells. The original flask contained motile merozoites at 102 days PI when part of the culture was cryopreserved and subcultured.

Protozoa were grown in EK cells inoculated with brain homogenate of mouse no. 4551 (culture B). Schizonts and merozoites were visible at 110 days when part of the culture was frozen in liquid nitrogen and the original flask was used

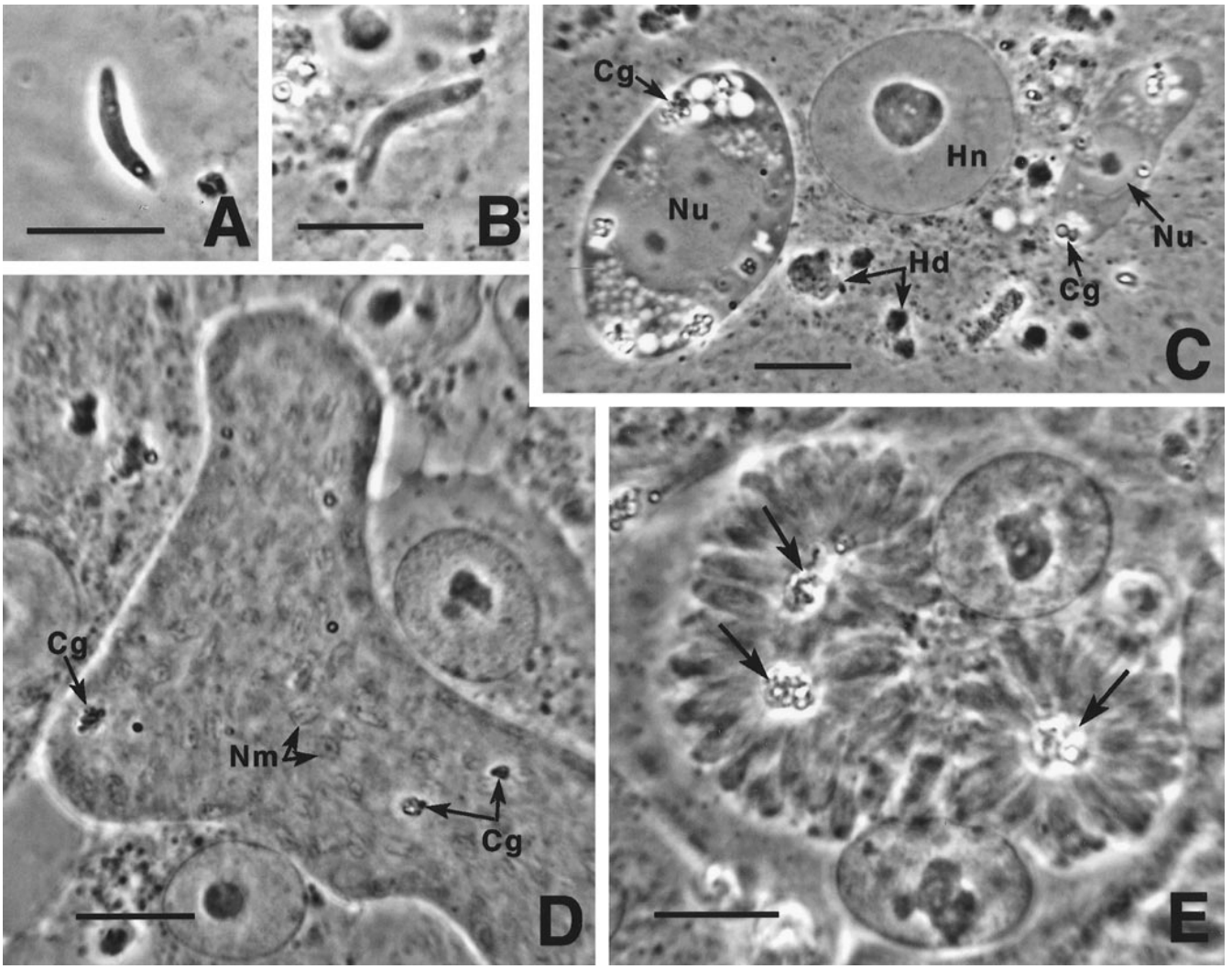

FIGURE 3. Phase-contrast photomicrographs of *S. speeri* in cultured equine kidney cells at 115 days of cultivation. Bars = 10 μm. **A.** Extracellular merozoite showing refractile bodies located at each pole. **B.** Intracellular merozoite immediately after penetrating a cultured cell. **C.** Two early schizonts in a host cell with a nucleus (Hn) with a prominent nucleolus. The schizont on the left has a nucleus (Nu) with several nucleoli; the other schizont has a smaller nucleus with a single nucleolus. Both schizonts have prominent cytoplasmic granules (Cg). Also note that the host cell cytoplasm contains debris of variable sizes (Hd). **D.** Schizont with merozoites budding internally. Each merozoite contains a single nucleus (Nm); cytoplasmic granules (Cg). **E.** Mature schizont in a binucleate host cell, showing merozoites in final stages of budding from 3 residual bodies (arrows).

for phase-contrast microscopy in double coverslip preparations.

Development in fixed coverslip cultures

Sarcocystis speeri merozoites entered CV-1 cells and underwent schizogony within 3 days (Fig. 2). Merozoites were usually located next to the host cell nucleus. Multiple penetration of a single host cell was observed at 1, 2, and 3 days PI and apparently at day 4 PI. Development and schizont nuclear division were asynchronous, resulting in cells containing structurally different organisms. Intracellular merozoites measuring 5.6 × 2.0 μm (4.0–7.2 × 1.6–3.2 μm, n = 20) were observed 1 day PI. A few developing merozoites measuring 8.7 × 3.9 μm (8.0–9.6 × 3.2–4.0 μm, n = 8) were also present at 1 day PI. The nucleus of these developing merozoites was enlarged, spherical, centrally located, and usually contained a prominent nucleolus (Fig. 2A). These developing merozoites grew in size and were 12.1 × 5.3 μm (8.0–16.8 × 3.2–7.2 μm, n = 20) 2 days PI. Developing schizonts with an enlarged nucleus containing several prominent nucleoli or a large lobulated nucleus were also present 2 days PI (Fig. 2B–D). These developing schizonts were 18.6 × 9.9 μm (16.0–24.0 × 6.4–14.4 μm, n = 20). By 3 days PI, the nucleus of schizonts underwent additional transformations to form grooves, lobes, and projections, and eventually portions of the nucleus were incorporated into merozoites as a single nucleus (Fig. 2D, E). These nearly mature schizonts were 29.9 × 17.0 μm (24.8–36.0 × 14.4–20.0 μm, n = 20) 3 days PI, and similar schizonts were 30.2 × 16.9 μm (24.0–36.0 × 14.4–20.0 μm, n = 20) 4 days PI. Merozoites in mature schizonts were 5.4 × 1.5 μm (4.8–6.4 × 0.2–2.0 μm,

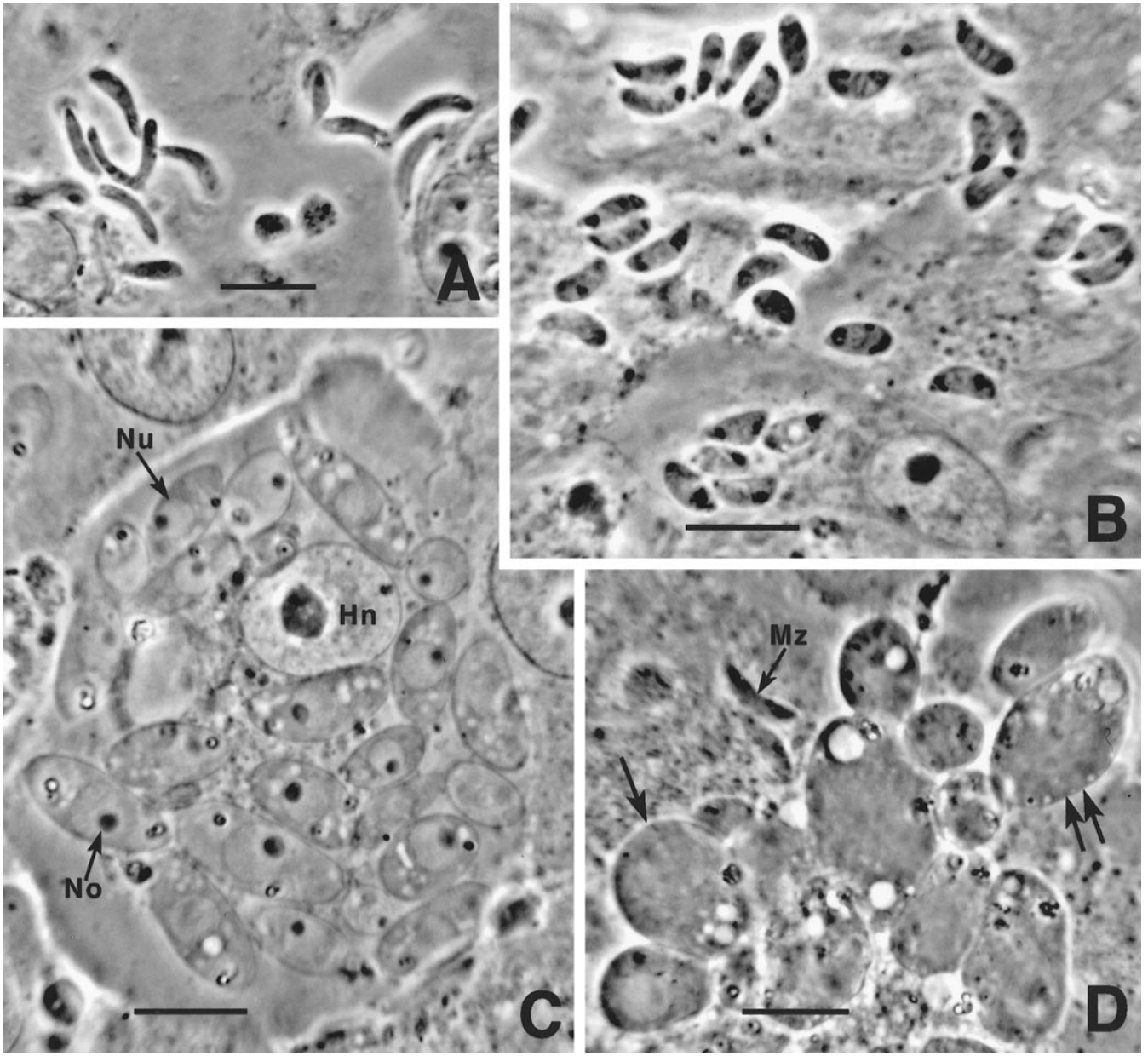

FIGURE 4. Phase-contrast photomicrographs of *S. speeri* in cultured kidney cells at 112 days of cultivation. Bars = 10 μm. **A.** Slender extracellular merozoites immediately after escape from host cell. **B.** Fatter intracellular merozoites at 20 min after entering cultured cells. **C.** Host cell containing approximately 23 early schizonts that were formed by merozoites that remained within the same host in which they had developed. Each schizont contains a nucleus (Nu) with a prominent nucleolus (No). **D.** Intracellular (single arrow) and extracellular (double arrow) oval-shaped intermediate schizonts that are being released from a lysed host cell. Mz, merozoite.

n = 20) 3 days PI and 5.6 × 1.6 μm (4.0–7.2 × 1.2–2.0 μm, n = 20) 4 days PI. Their nuclei were either central or slightly posterior in location. Merozoites budded at the surface of the schizont prior to cytokinesis. A residual body was occasionally seen (Fig. 2G). On occasion, both slender and stubby merozoites were present together (Fig. 2H, I).

Growth in live double coverslip preparations

In double coverslip preparations, extracellular merozoites were slender, measuring 10.3 × 2.8 μm (10–11 × 2.5–3 μm; n = 20) (Figs. 3A, 4A). Within a few minutes after penetrating cultured cells, merozoites transformed from slender (Fig. 3B) into broader merozoites (Fig. 4B) measuring 6.4 × 3.2 μm (6–7.5 × 3–4 μm; n = 20). Merozoites transformed into oval-shaped schizonts that contained a large spheroidal nucleus with a prominent nucleolus and cytoplasmic granules and vacuoles (Figs. 3C, 4C). As the schizont developed, its single nucleus became larger and irregular in shape and contained several nucleoli varying in size from 0.25 to 1.0 μm. In more advanced intermediate schizonts, the nucleus and nucleoli became indistinct. In maturing schizonts, multiple merozoite buds and their nuclei could be seen internally as well as several concentrations

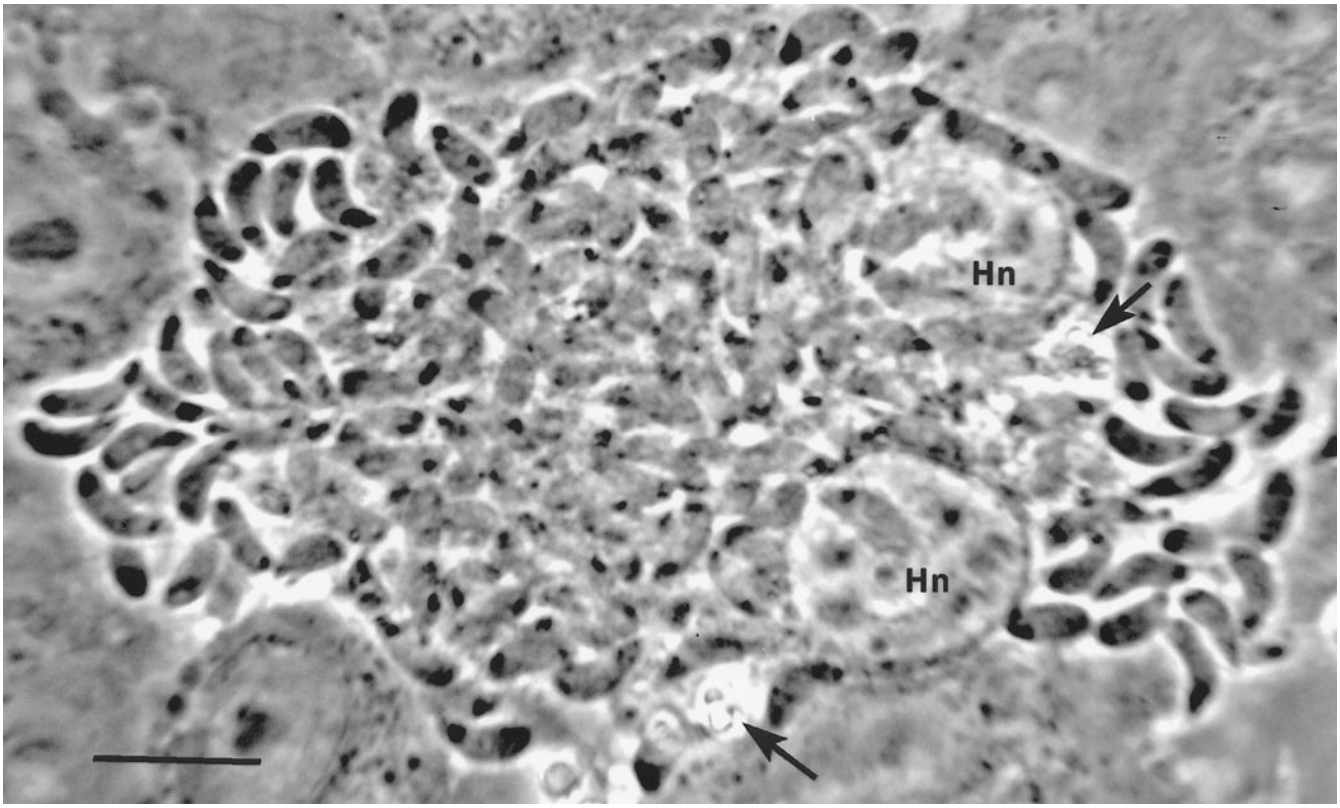

FIGURE 5. Phase-contrast photomicrograph of *S. speeri* merozoites escaping from a multiply-infected host cell with 2 nuclei (Hn) at 115 days of culturation. Note schizont residual bodies (arrows). Bar = 10 μm.

of cytoplasmic granules (Fig. 3D). Mature schizonts contained merozoites that budded radially from 2 to 6 residual bodies. The cytoplasmic granules seen in intermediate schizonts became concentrated in the schizont's residual bodies (Fig. 3E). Mature schizonts were 42 × 28 μm (30–55 × 20–35 μm; n = 20) and contained 86 (50–120; n = 20) merozoites. Some merozoites escaped from their host cells, entered other cells, and repeated schizogony, but most of them remained within the host cell in which they had developed and began to undergo schizogony. Some of these merozoites reached maturity that resulted in some host cells containing numerous merozoites (Fig. 5). The larger schizonts, containing as many as 120 merozoites, may have actually developed from host cells containing 2 or more schizonts (Fig. 5). However, of the multiply-infected cells lysed, releasing merozoites and schizonts in various stages of development (Fig. 3D), extracellular oval-shaped schizonts were nonmotile, did not penetrate into other cultured cells, and eventually degenerated. Most of the schizonts within multiply-infected host cells degenerated and did not form mature schizonts.

Infectivity of *S. speeri* merozoites to mice

Mice inoculated with *S. speeri* merozoites remained healthy. *Sarcocystis* stages were not found in tissues of 5 KO mice injected with *S. speeri*-infected liver homogenates; these mice were killed 78 (2 mice) and 99 (3 mice) days PI. Similar results were obtained in 5 KO mice injected with cultured merozoites. These mice were killed 35 (2 mice), 42 (1 mouse), and 85 (2 mice) days PI. However, in another KO mouse killed 42 days PI, few merozoites were seen in an inflammatory focus in the brain; this mouse was injected with merozoites from culture A.

Infectivity of *S. speeri* merozoites to budgerigars

Schizonts or sarcocysts of *Sarcocystis* were not demonstrable in tissues of budgerigars infected with cultured *S. speeri* merozoites.

DISCUSSION

As stated earlier, at least 3 species of *Sarcocystis, S. neurona, S. falcatula,* and *S. speeri,* have been recognized in the North American opossum, *D. virginiana* (reviewed in Dubey and Lindsay, 1999) and *S. falcatula* and *S. speeri* from the South American opossum, *D. albiventris* (Dubey, Venturini, Venturini, and Speer, 2000; Dubey, Venturini, Venturini, Basso, and Unzaga, 1999; Dubey, Speer et al., 2000). Of these, *S. falcatula* was cultured in vitro from lungs of budgerigars that had been fed sporocysts from the opossum *D. virginiana* from the U.S. (Marsh et al., 1997; Lindsay et al., 1999) and *D. albiventris* from Argentina (Dubey, Venturini, Venturini, Basso, and Unzaga, 1999; Dubey, Lindsay et al., 2000). *Sarcocystis neurona* was first cultured in vitro from the spinal cord of paralyzed horses in 1991 (Dubey et al., 1991; Davis et al., 1991) and from sporocysts from the opossum intestines in 1998 (Dubey

and Lindsay, 1998); *S. neurona* has not yet been cultured from either horses or opossums in South America.

In the present study the donor opossum, *D. albiventris,* used to cultivate *S. speeri* was from Argentina (Dubey, Venturini, Venturini, and Speer, 2000). This opossum also had *S. falcatula* as determined by bioassay in budgerigars (Dubey, Venturini, Venturini, Basso, and Unzaga, 1999) but not *S. neurona* as determined by bioassay in KO mice. *Sarcocystis speeri,* although originally described from *D. virginiana* (Dubey and Lindsay, 1999) has not yet been cultivated in vitro from this host. Thus, the present cultivation of *S. speeri* was from the Argentinian opossum, *D. albiventris.*

Results of the present study indicate that the parasite cultivated from the tissues of mice is *S. speeri* and not *S. neurona* based on morphology and antigenicity. As stated earlier, at least 3 species of *Sarcocystis, S. neurona, S. falcatula,* and *S. speeri* are present in opossums in North America. Schizonts and merozoites of *S. falcatula* and *S. neurona* in cell culture are similar in size but divide differently; residual bodies are prominent in *S. neurona* schizonts but indistinct or rare in *S. falcatula* schizonts (Lindsay et al., 1999; Speer et al., 2000). In *S. speeri* schizonts, residual bodies were rare in Giemsa-stained smears but were visible in double coverslip, phase-contrast preparations. *Sarcocystis speeri* merozoites are morphologically distinct from *S. neurona* and *S. falcatula* merozoites; *S. speeri* merozoites are slender and longer than those of *S. falcatula* and *S. neurona.*

In the present study both elongated and stubby merozoites were seen in smears made from infected tissues and in in vitro-cultured schizonts. Whether these merozoites belonged to different generations of schizogony was not determined. In double coverslip studies of *S. speeri* in cultured cells, extracellular merozoites were elongated, whereas those that entered cells became shorter. Thus, the stubby merozoites may have resulted from merozoites being released from their host cells during smear preparation. *Sarcocystis speeri* multiplied in the same flask up to 110 days. The development of *S. speeri* in cultured cells was similar to that found for *S. neurona* and *S. falcatula* (Speer et al., 1999) in which the parasites underwent multiple generations of schizogony and merozoites often remained within the original host cell in which they had developed and continued development into schizonts. Thus, multiple schizogonous generations occurred within the same host cell.

As reported earlier, *S. speeri* schizonts did not stain with anti-*S. neurona* antibodies and *S. neurona* schizonts did not react with anti-*S. speeri* antibodies (Dubey and Lindsay, 1999). Cell culture-derived *S. speeri* merozoites were used to obtain anti-*S. speeri* antibodies (Dubey and Lindsay, 1999).

Merozoites of *S. speeri* grown in vitro are biologically distinct from those of *S. neurona* and *S. falcatula. Sarcocystis speeri* merozoites were not infective to budgerigars or to KO mice. The lack of infectivity of *S. speeri* merozoites from culture or infected tissues to KO mice indicates that, unlike *S. falcatula* and *S. neurona, S. speeri* likely has an obligatory 2-host cycle and a narrow host range. *Sarcocystis neurona* merozoites from cell culture and tissues are infective to immunosuppressed mice (Marsh et al., 1997; Dubey and Lindsay, 1998), and *S. neurona*-like infections have been reported in a variety of mammals including raccoons, mink, monkeys, and skunk. *Sarcocystis falcatula* merozoites are infective to birds, and the parasite has been reported in several species of birds (Box et al., 1984; Marsh et al., 1997; Dubey and Lindsay, 1998). The natural intermediate host for *S. speeri* is unknown. The successful in vitro cultivation of *S. speeri* should be helpful in producing *S. speeri* specific diagnostic reagents.

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